

Phagocytosis genes nonautonomously promote developmental cell death in the *Drosophila* ovary

Allison K. Timmons^a, Albert A. Mondragon^a, Claire E. Schenkel^a, Alla Yalonetskaya^a, Jeffrey D. Taylor^a, Katherine E. Moynihan^a, Jon Iker Etchegaray^a, Tracy L. Meehan^a, and Kimberly McCall^{a,1}

^aDepartment of Biology, Boston University, Boston, MA 02215

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Programmed cell death (PCD) is usually considered a cell-autonomous suicide program, synonymous with apoptosis. Recent research has revealed that PCD is complex, with at least a dozen cell death modalities. Here, we demonstrate that the large-scale nonapoptotic developmental PCD in the *Drosophila* ovary occurs by an alternative cell death program where the surrounding follicle cells nonautonomously promote death of the germ line. The phagocytic machinery of the follicle cells, including Draper, cell death abnormality (Ced)-12, and c-Jun N-terminal kinase (JNK), is essential for the death and removal of germ-line-derived nurse cells during late oogenesis. Cell death events including acidification, nuclear envelope permeabilization, and DNA fragmentation of the nurse cells are impaired when phagocytosis is inhibited. Moreover, elimination of a small subset of follicle cells prevents nurse cell death and cytoplasmic dumping. Developmental PCD in the *Drosophila* ovary is an intriguing example of nonapoptotic, nonautonomous PCD, providing insight on the diversity of cell death mechanisms.

Drosophila | ovary | cell death | phagocytosis | engulfment

Programmed cell death (PCD) is the genetically controlled elimination of cells that occurs during organismal development and homeostasis. Cells are considered dead when they have undergone irreversible plasma membrane permeabilization or have become completely fragmented (1). Apoptosis is the most well-characterized form of PCD, however there are at least a dozen cell death modalities that are morphologically, biochemically, and genetically distinct (2, 3). Two examples of nonapoptotic cell death are autophagic cell death and necrosis, but there are several alternative cell death mechanisms that are less well understood.

Nonapoptotic PCD occurs on a large scale in the *Drosophila* ovary. *Drosophila* females can produce hundreds of eggs during their lifetime, and for every egg that is formed, developmental PCD of supporting nurse cells (NCs) occurs. However, the mechanisms of developmental PCD in the *Drosophila* ovary are poorly understood. Each egg forms from a 16-cell germ-line cyst, comprised of the single oocyte and 15 NCs that support the oocyte throughout 14 stages of oogenesis (4, 5). Hundreds of somatically derived follicle cells (FCs) surround the germ-line cyst, forming an egg chamber. At stage 11 of oogenesis, NCs rapidly transfer (“dump”) their cytoplasm into the oocyte. Concurrently, the NCs asynchronously undergo developmental PCD, resulting in mature stage 14 egg chambers that no longer contain any NCs (4–6). Interestingly, caspases, proteases associated with apoptosis, play only a minor role in the death of the NCs in late oogenesis (7–9). Furthermore, combined inhibition of caspases and autophagy does not significantly block NC death during late oogenesis (10). To date, defining the major mechanism of developmental PCD in the *Drosophila* ovary has remained elusive.

An intriguing possibility is that the somatic FCs non-cell-autonomously promote developmental PCD of the NCs during late oogenesis. Non-cell-autonomous regulation of PCD occurs when a cell or group of cells extrinsically initiates or promotes the death of another cell. This concept challenges the idea that PCD is largely a self-regulated, autonomous suicide program in which a cell controls its own demise. One well-characterized example of

non-cell-autonomous control of PCD is apoptosis induced by the death ligands Fas or TNF (11, 12).

Another type of non-cell-autonomous PCD is phagoptosis (or primary phagocytosis), in which engulfing cells directly cause the death of other cells via “murder” or “assisted suicide.” Phagoptosis is distinct from the engulfment of cell corpses, as the engulfing cell plays an active role in the death of a cell, rather than simply degrading a cell that died via another mechanism. The defining characteristic of phagoptosis is that inhibition of phagocytosis leads to a failure in cell death (13, 14). Phagoptosis has been demonstrated in activated microglia that phagocytose viable neurons, resulting in their destruction (13–15). Entosis is another example of non-cell-autonomous PCD, often referred to as “cell cannibalism,” in which a viable cell invades another cell, where it is degraded by lysosomes. Entosis is distinct from phagoptosis, as the inhibition of phagocytosis genes does not prevent entosis (16). Phagocytosis has also been shown to promote PCD in *Caenorhabditis elegans*, although this is an example of assisted suicide, as dying cells also require apoptotic machinery (17, 18).

Genetic studies in *C. elegans* have identified two partially redundant signaling pathways that control phagocytosis: the cell death abnormality (CED)-1, 6, 7 and CED-2, 5, 12 pathways (19–21). The CED-1, 6, 7 and CED-2, 5, 12 pathways act in parallel to promote the activation of CED-10, a Rac GTPase responsible for cytoskeletal rearrangements that allow for internalization of the cell corpse. In *Drosophila*, the roles of the *Ced-1*, 6, 7 and *Ced-2*, 5, 12 pathways appear to be conserved. The CED-1 ortholog, Draper, is a transmembrane protein that localizes to the surface of the engulfing cell and acts as a receptor to recognize dying cells. Draper was first shown to be required for engulfment of apoptotic neurons in the embryonic central nervous system with mutants displaying lingering cell corpses (22). Additionally, Draper has been shown to be

Significance

Programmed cell death is usually considered a cell-autonomous suicide program, synonymous with apoptosis. Here we demonstrate that a specific example of large-scale nonapoptotic developmental programmed cell death in the *Drosophila* ovary occurs by an alternative cell death program where surrounding epithelial cells nonautonomously promote the death of the germ line. We find that genes normally required for engulfment of dying cells act to promote the death of the germ line. Developmental programmed cell death in the *Drosophila* ovary is an intriguing example of nonapoptotic, nonautonomous cell death, providing insight on the diversity of cell death mechanisms.

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¹To whom correspondence should be addressed. Email: kmccall@bu.edu.

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important in several other contexts including the engulfment of severed axons, bacteria, imaginal disc cells, hemocytes, and apoptotic NCs in midoogenesis (23–27). In addition to Draper, other *Drosophila* engulfment receptors include Croquemort (28) and integrins (29–31). Croquemort is related to CD36, a scavenger receptor involved in engulfment in mammals (32), and integrins also act as engulfment receptors in *C. elegans* and mammals (33, 34). The upstream activators of the Ced-2, 5, 12 pathway are largely unknown, although integrins may activate the pathway (34). As in *C. elegans*, it appears that *Ced-12* and *draper* function in separate pathways in *Drosophila*. *Ced-12* and *draper* have been shown to function in distinct steps in axon clearance (35). In macrophages, *Ced-12* has been shown to function in a separate pathway from *simu*, a bridging molecule that acts upstream of *draper* (36). A number of other engulfment genes have been identified in *Drosophila*, and their molecular interactions are under active investigation (36–39).

Given the minor role for apoptosis and autophagic cell death during developmental PCD in the *Drosophila* ovary, we investigated the possibility that the FCs non-cell-autonomously promote NC death. Previously we showed that FCs of the *Drosophila* ovary are capable of phagoptosis in midoogenesis when phagocytosis genes are overexpressed (27), and we questioned whether phagocytosis genes might normally function to control cell death in late oogenesis. Indeed, we found that the phagocytosis genes *draper* and *Ced-12/ELMO* are required in the FCs for NC removal in late oogenesis and that they function partly in parallel. We also show that the FCs non-cell-autonomously control events associated with the death of the NCs, including nuclear envelope permeabilization, acidification, and DNA fragmentation. Furthermore, the genetic ablation of stretch FCs disrupted all cellular changes associated with developmental PCD of the NCs. Therefore, PCD of the NCs is a unique model

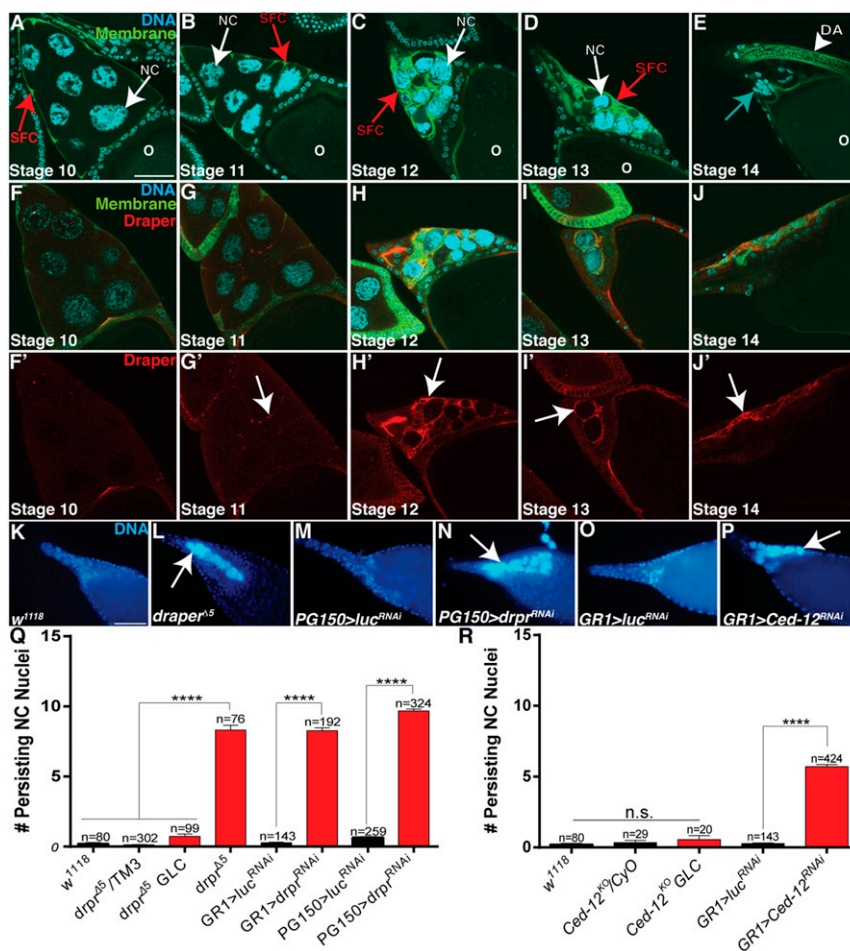


Fig. 1. *draper* is required in the FCs for the removal of NCs during late oogenesis. (A–E) The stretch FCs surround the NCs during late oogenesis. Stage 10–14 egg chambers (*PG150-GAL4/+; UAS-mCD8-GFP/+*) express GFP specifically in the membranes of the stretch FCs (green, red arrows) and are stained with DAPI to label DNA (cyan). NC nuclei are indicated with white arrows. Oocyte is labeled with “O.” (A) Stretch FCs are apparent on the anterior of a stage 10 egg chamber. (B) Stretch FCs begin to extend around NCs in stage 11. (C) Stretch FCs surround NC nuclei in stage 12. (D) Stretch FCs continue to surround NC nuclei in stage 13. (E) A stage 14 egg chamber no longer contains NC nuclei and has fully formed DAs (arrowhead). Small nuclei are FC nuclei (blue arrow). (F–J’) *Draper* is enriched on the FC membranes (arrows) in late oogenesis. Stage 10–14 egg chambers (*UAS-mCD8-GFP/+; GR1-GAL4/+*) are stained with α -*Draper* antibody (red) and DAPI (cyan) and express GFP specifically in the FC membranes (green). (F and F’) Stage 10 egg chamber has nonspecific staining along the NC/oocyte interface. (G and G’) *Draper* enrichment becomes apparent in stage 11. (H and H’) Stage 12 egg chamber has enriched *Draper* on the FC membranes. (I and I’) Stage 13 egg chamber with *Draper* enrichment on the FC membranes. (J and J’) Stage 14 egg chamber has residual *Draper* staining. (Scale bar, 50 μ m for images A–J’.) (K–P) Persisting NC nuclei (arrows) are present in stage 14 egg chambers with engulfment genes knocked down. Stage 14 egg chambers were stained with DAPI (blue). (Scale bar, 20 μ m.) (K) Wild type (*w¹¹¹⁸*). (L) Homozygous *draper^{Δ5}*. (M) Control (*PG150-GAL4/+; UAS-luciferase^{RNAi/+}*). (N) *draper^{RNAi}* expressed specifically in the stretch FCs (*PG150-GAL4/+; UAS-draper^{RNAi/+}*). (O) Control (*GR1-GAL4/UAS-luciferase^{RNAi}*). (P) *Ced-12^{RNAi}* expressed specifically in the FCs (*GR1-GAL4/UAS-Ced-12^{RNAi}*). (Q and R) Quantification of persisting NC nuclei in stage 14 egg chambers. GLC, germ-line clone. Data from two distinct RNAi lines were combined for *Ced-12*. Data presented are mean \pm SEM. **** $P \leq 0.0001$.

of a naturally occurring developmental cell death program that is nonapoptotic and non-cell-autonomously controlled.

Results

Stretch FCs Surround NCs During Late Oogenesis. During late oogenesis, a population of ~50 FCs known as the stretch FCs cover the NCs on the anterior of the egg chamber, and it has been proposed that these FCs phagocytose the NCs following their death (40–42). However, exactly how this is accomplished and whether stretch FCs play a role in the developmental PCD of the NCs remains unclear. To analyze the relationship between stretch FCs and NCs, we expressed a membrane-tethered GFP specifically in stretch FCs (*PG150 > mCD8-GFP*) (Fig. 1 *A–E* and Fig. S1*A*). The stretch FCs were visible in stage 10 (Fig. 1*A*) and began to project extensions around individual NCs in stage 11 (Fig. 1*B*). By stage 12, cytoplasmic dumping of the NCs was completed, and the NC nuclei appeared completely enveloped by the stretch FCs (Fig. 1*C*). The stretch FCs remained around the NC nuclei in stage 13, as they were eliminated (Fig. 1*D*). By stage 14, characterized by fully formed dorsal appendages (DAs), all NC nuclei were eliminated (Fig. 1*E*). Therefore, the stretch FCs were intimately associated with the NCs throughout the progression of developmental PCD, raising the intriguing possibility that the stretch FCs play an active role in the death of the NCs.

***draper* Is Required Specifically in the Stretch FCs for NC Removal.** Given that the stretch FCs completely surrounded the NCs throughout late oogenesis, we investigated whether the phagocytic machinery in the FCs contributed to NC removal. Egg chambers expressing a membrane-tethered GFP specifically in all FCs (*GRI > mCD8-GFP*; Fig. S1*B*) were stained with an antibody against the engulfment receptor Draper (Fig. 1 *F–J*). Draper was detected as the stretch FCs surrounded the NCs in stage 11 and appeared to define the path of stretch FC extension (Fig. 1 *G* and *G'*). The enrichment of Draper on the FC membranes was most intense in stages 12 and 13 (Fig. 1 *H–I*), and some residual Draper staining was observed in stage 14 (Fig. 1 *J* and *J'*). The enrichment of Draper was specific to the stretch FCs, because egg chambers with *draper* knocked down specifically in stretch FCs (*PG150 > draper^{RNAi}*) lacked Draper staining in late stage egg chambers (Fig. S1 *D–G*).

To determine whether *draper* was required for the removal of NCs during late oogenesis, we analyzed egg chambers from *draper^{Δ5}* (null) flies. Interestingly, we found a striking number of persisting NC nuclei in stage 14 egg chambers (Fig. 1*L*), compared with the control (*w¹¹¹⁸*), where NC nuclei were removed normally (Fig. 1*K*). On average, there were ~8 persisting NC nuclei in *draper^{Δ5}* egg chambers compared with 0.23 in the *w¹¹¹⁸* control (Fig. 1*Q*). Moreover, 100% of *draper^{Δ5}* stage 14 egg chambers contained at least one persisting NC nucleus, and >40% had more than 10 persisting nuclei (Fig. S1*C*). These data show that *draper* is required for the removal of the NCs.

draper has been shown in several contexts to be required in engulfing cells for corpse clearance (22–27). However, *draper* was also shown to be required cell-autonomously in the salivary gland for autophagic cell death (43). To determine which cell type required *draper* during developmental PCD of the NCs, we generated *draper^{Δ5}* germ-line clones (GLCs) and found that the number of persisting NC nuclei was significantly reduced compared with *draper^{Δ5}* homozygotes (Fig. 1*Q* and Fig. S1*C*), suggesting that *draper* is required nonautonomously for NC removal. Next, we expressed *draper^{RNAi}* using FC-specific GAL4 drivers (Fig. S1 *A* and *B*). Compared with controls (Fig. 1 *M* and *O*), stage 14 egg chambers expressing *draper^{RNAi}* in all FCs (*GRI > draper^{RNAi}*) had a strong persisting NC nuclei phenotype similar to *draper^{Δ5}* (Fig. 1*Q* and Fig. S1*C*). Furthermore, we demonstrated that the requirement for *draper* was specifically in the stretch FCs (*PG150 > draper^{RNAi}*) (Fig. 1 *M*, *N*, and *Q*), with

~55% of egg chambers containing >10 persisting NC nuclei (Fig. S1*C*). Together, these findings indicate that *draper* is specifically required in the stretch FCs for NC removal in late oogenesis.

To identify other genes that are required in the FCs for NC removal, we knocked down several candidate genes specifically in the FCs via RNAi or dominant-negative constructs (Table S1). The candidate screen revealed that FC-specific knockdown of several known engulfment genes, including *Ced-12*, resulted in persisting NC nuclei (Fig. 1 *O*, *P*, and *R* and Fig. S1 *H* and *I*), further demonstrating that the engulfment machinery of the FCs is important for the removal of NCs during late oogenesis. To determine if *Ced-12* was required in the germ line, we generated GLCs and found that the number of persisting NC nuclei was not significantly different from the *w¹¹¹⁸* control, demonstrating that *Ced-12* is required nonautonomously in the FCs for NC removal (Fig. 1*R* and Fig. S1*H*). Although both *draper* and *Ced-12* knockdowns demonstrated a severe disruption to the removal of the NCs, most of the FCs still appeared to surround the NCs, indicating that the FCs were morphologically normal (Fig. S1 *J–L*). Several other known engulfment genes, including integrins, *Gprk2*, *mbc*, *shark*, *Src42A*, and *Rac1*, were found to disrupt NC removal when knocked down in the FCs (Table S1 and Fig. S1*I*).

***draper* and *Ced-12* Act in Parallel to Promote Developmental PCD of the NCs.** In *C. elegans*, it has been reported that *Ced-12* acts in parallel to *Ced-1*, the ortholog of *draper* (20, 44), and evidence in *Drosophila* suggests that this may be conserved (35, 36). To determine whether *draper* and *Ced-12* functioned in parallel in FCs, we performed double mutant analysis and found that *draper^{RNAi} Ced-12^{RNAi}* double knockdowns had a more severe persisting nuclei phenotype than either *draper* or *Ced-12* alone (Fig. 2 *A–D*). Over 20% of stage 14 egg chambers had 13–15 persisting nuclei in *draper^{RNAi} Ced-12^{RNAi}* double knockdowns (Fig. 2*D*), the strongest phenotype we encountered. To determine if this interaction could be due to incomplete knockdowns with the RNAi, we repeated this experiment using the null allele, *draper^{Δ5}*, in combination with *Ced-12^{RNAi}* and observed a similar enhanced phenotype (Fig. 2 *C* and *D*). Although the majority of NCs were affected, the knockdown of *draper* and *Ced-12* did not cause a complete disruption to the removal of NCs, raising the possibility that a third pathway (autonomous or non-cell-autonomous) may also contribute. The incomplete disruption to NC removal could also be due to incomplete knockdown of *Ced-12* via RNAi. Together, these data suggest that *draper* and *Ced-12* act in parallel pathways to promote NC removal.

The Accumulation of NC Nuclei Inhibits Egg Laying. We observed a striking accumulation of NC nuclei in the ovaries (Fig. 2 *E* and *F*) of mutants with strong persisting NC nuclei phenotypes. We questioned whether the accumulation of NC nuclei in the ovary could affect the fecundity of the flies. Initially, we tested *draper Ced-12* double knockdowns in all FCs because they had the strongest phenotype and found that both the *draper Ced-12* double knockdown and the *Ced-12* knockdown were completely sterile (Fig. S2*A*). However, this was likely due to a requirement for *Ced-12* during border cell migration (Fig. S2 *B–D*) (45). Interestingly, we also noticed that *draper^{Δ5}* mutants displayed defects in border cell migration (Fig. S2 *E–G*). To avoid the possibility that border cell migration could disrupt egg laying, we used *PG150-GAL4* to knock down *draper* and *Ced-12* only in the stretch FCs. We confirmed that *Ced-12* did not cause a border cell migration defect when knocked down in the stretch FCs (Fig. S2*H*). When we measured egg laying in *draper^{RNAi} Ced-12^{RNAi}* double knockdowns, we found that females laid fewer eggs per day than the control (Fig. 2*G*). In an independent experiment, we observed defects in egg laying in *draper^{RNAi}* and *Ced-12^{RNAi}* single knockdowns (Fig. S2*J*). Therefore, when phagocytosis genes were inhibited in the FCs, NC nuclei accumulated in the ovary and there was an adverse effect on fecundity.

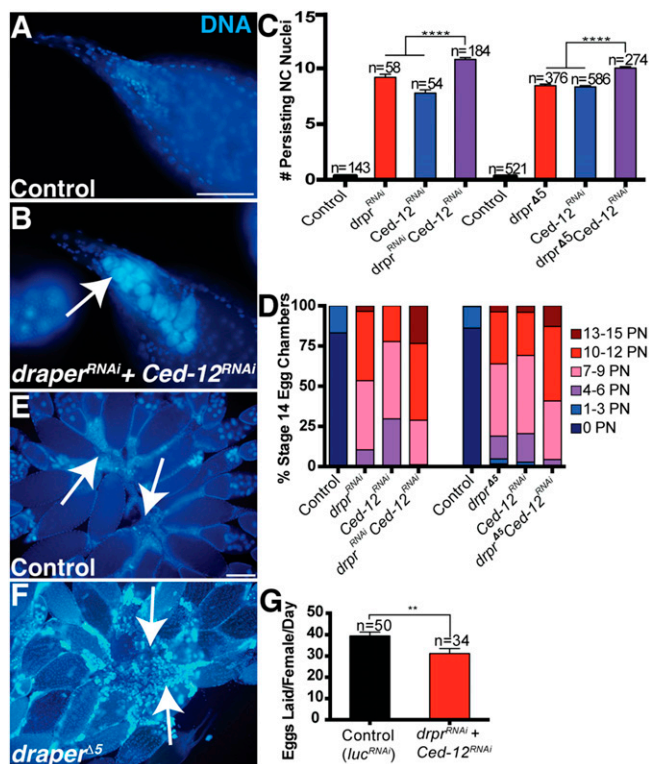


Fig. 2. *Ced-12* and *draper* act in parallel pathways, and mutants show reduced fecundity. (A and B) *draper*^{RNAi} *Ced-12*^{RNAi} double knockdowns have a severe persisting nuclei phenotype (arrow). Stage 14 egg chambers have a severe persisting nuclei phenotype (arrow). Stage 14 egg chambers are stained with DAPI (blue). (Scale bar, 20 μ m.) (A) Control (*UAS-GAL4/Sco; MKRS/TM6B*) stage 14 egg chamber does not have any persisting NC nuclei. (B) Egg chamber from *draper*^{RNAi} *Ced-12*^{RNAi} double knockdown (*UAS-GAL4/UAS-draper*^{RNAi}; *GR1-GAL4 G89/UAS-Ced-12*^{RNAi}) exhibits a complete failure in NC removal with 15 persisting NC nuclei. (C) Quantification of persisting NC nuclei in stage 14 egg chambers. *draper*^{RNAi} *Ced-12*^{RNAi} knockdown (purple, *UAS-GAL4/UAS-draper*^{RNAi}; *GR1-GAL4 G89/UAS-Ced-12*^{RNAi}) has a more severe persisting nuclei phenotype than the *draper*^{RNAi} knockdown (red, *UAS-GAL4/UAS-draper*^{RNAi}; *GR1-GAL4 G89/MKRS*) or *Ced-12*^{RNAi} knockdown (blue, *UAS-GAL4/CyO; GR1-GAL4 G89/UAS-Ced-12*^{RNAi}). *draper*^{Δ5} *Ced-12*^{RNAi} double knockdown (purple, *draper*^{Δ5} *UAS-Ced-12*^{RNAi}/*draper*^{Δ5} *GR1-GAL4*) has a more severe persisting NC nuclei phenotype than *draper*^{Δ5} (red, *draper*^{Δ5} *UAS-Ced-12*^{RNAi}/*draper*^{Δ5}) or *Ced-12*^{RNAi} (blue, *draper*^{Δ5} *UAS-Ced-12*^{RNAi}/*GR1-GAL4 UAS-mCD8-GFP*) single knockdowns. Mixed sibling controls lack either the *GAL4* driver or the RNAi constructs. Data presented are mean \pm SEM. **** $P \leq 0.0001$. (D) Alternative quantification of data presented in Fig. 2C (see *Materials and Methods*). The *draper* *Ced-12* double knockdowns result in a stronger persisting nuclei phenotype than *draper* or *Ced-12* single knockdowns. (E and F) Intact ovaries stained with DAPI (blue). (Scale bar, 100 μ m.) (E) Control [*FRT 2A/Df(3L)BSC181*] ovary does not contain lingering NC nuclei. The center of the ovary contains FCs that have been shed from mature stage 14 egg chambers as they enter the oviduct (arrows). (F) NC nuclei accumulate in homozygous *draper*^{Δ5} ovary (arrows). (G) *draper*^{RNAi} *Ced-12*^{RNAi} double knockdowns in stretch FCs (*PG150-GAL4/+; UAS-GAL4/UAS-draper*^{RNAi}; +/*UAS-Ced-12*^{RNAi}) have reduced egg laying compared with the control (*PG150-GAL4/+; UAS-GAL4/+; UAS-luciferase*^{RNAi/+}). Egg laying was quantified in females 21–25 d old. *n*, number of females. Data presented are average number of eggs laid per female per day \pm SEM. ** $P \leq 0.01$.

Lysosomal and Intracellular Trafficking Genes Are Required in the FCs for NC Removal. Previous work has demonstrated a role for lysosomal trafficking genes such as *deep orange* (*dor*) in developmental NC death (46). *dor* is a member of the HOPS complex that localizes to endosomes and is important for delivery of material to lysosomes (47). As we previously reported (46), *dor* hypomorphs have defects in NC removal (Fig. 3 A, B, and E). Interestingly, we found that the requirement for *dor* is in the FCs. *dor* GLCs largely

showed normal NC removal (Fig. 3 C and E), and the expression of *dor*^{RNAi} specifically in the FCs (*GR1 > dor*^{RNAi}) resulted in persisting NC nuclei (Fig. 3 D and E). Intracellular trafficking genes—*Rab5*, *Rab7*, and *Rab35*—were also found to be required in the FCs for the elimination of the NCs (Fig. S34 and Table S1). To determine whether *draper* acts in the same pathway or in a parallel pathway to intracellular trafficking genes, we analyzed *draper*^{Δ5} *Rab35*^{RNAi} double mutants. We found that the double mutants were not significantly different from *draper*^{Δ5} (Fig. S3 B and C). This suggests that *draper* acts in the same pathway as intracellular trafficking proteins, like its ortholog *ced-1* (48).

***draper* and *Ced-12* Are Required in the FCs for the Acidification of the NCs.** We previously showed that punctate LysoTracker staining, likely labeling lysosomes, surrounds NC nuclei in late oogenesis and progresses to complete acidification of NC nuclei (46, 49). Using double labeling, we found that most LysoTracker puncta overlapped with the membranes of stretch FCs (Fig. 3 F and F'), raising the possibility that lysosomes from the stretch FCs play an active role in NC death. Electron microscopy has also demonstrated that lysosomes are present in the FCs that border the NCs, but not in FCs surrounding the oocyte, indicating that they may be important for NC elimination (40). By stages 12 and 13, the majority of wild-type egg chambers contained LysoTracker-positive nuclei and puncta (Fig. 3 G, G', and I). However, LysoTracker labeling in *draper*^{RNAi} and *Ced-12*^{RNAi} single knockdowns was severely reduced in stage 12–13 egg chambers compared with the control (Fig. 3 I). Strikingly, *draper*^{RNAi} *Ced-12*^{RNAi} double knockdowns showed a complete disruption to NC acidification (Fig. 3 H and I). Therefore, the engulfment machinery in the FCs is required for acidification of NCs during developmental PCD. However, whether the FC lysosomes actively contribute to the death process or are involved in the phagocytic processing of the NCs remains unknown.

***draper* Is Required in the Stretch FCs for Fragmentation of the NC Nuclei.** DNA fragmentation is an important step in the destruction of a cell and is considered to be a hallmark of apoptotic cell death (50). Typically, DNA fragmentation is executed by the autonomous activation of endonucleases, such as caspase-activated DNase (CAD), and is considered to be the “point of no return” in the death process (46, 51). TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining is used to label 3'-OH ends of fragmented DNA in apoptotic cells (52, 53). During developmental PCD of NCs in late oogenesis, NC nuclei become TUNEL-positive, indicating that DNA fragmentation occurs (41, 53–56). Consistent with previous findings, we observed that NC nuclei became TUNEL-positive, especially in stage 13 (Fig. 4 A, A', C, C', and E). Interestingly, TUNEL-labeled NC nuclei were often not detectable by DAPI. Quantification revealed that 35–50% of control stage 13 egg chambers (*nanos-GAL4/+* and *PG150 > luciferase*^{RNAi}) contained TUNEL-positive NC nuclei (Fig. 4E). Consistent with findings that developmental PCD of the NCs is largely caspase-independent (7–10), we found that overexpression of the caspase inhibitor *Diap1* in the NCs (*nanos > UASp-Diap1*) did not disrupt DNA fragmentation in late oogenesis (Fig. 4 B, B', and E). In contrast, we found that TUNEL staining was completely absent in stages 12 and 13 when *draper* was knocked down in the stretch FCs (Fig. 4 D and E). These data demonstrate that DNA fragmentation is inhibited when *draper* is knocked down in the FCs, implicating a role for the FCs in the death of the NCs.

***draper* and *Ced-12* Act in the FCs to Promote NC Nuclear Permeability During Developmental PCD.** To further investigate whether the FC engulfment machinery affected the death of the NCs, we examined permeability of the NC nuclear envelope, one of the earliest indications of developmental PCD (57, 58). To visualize

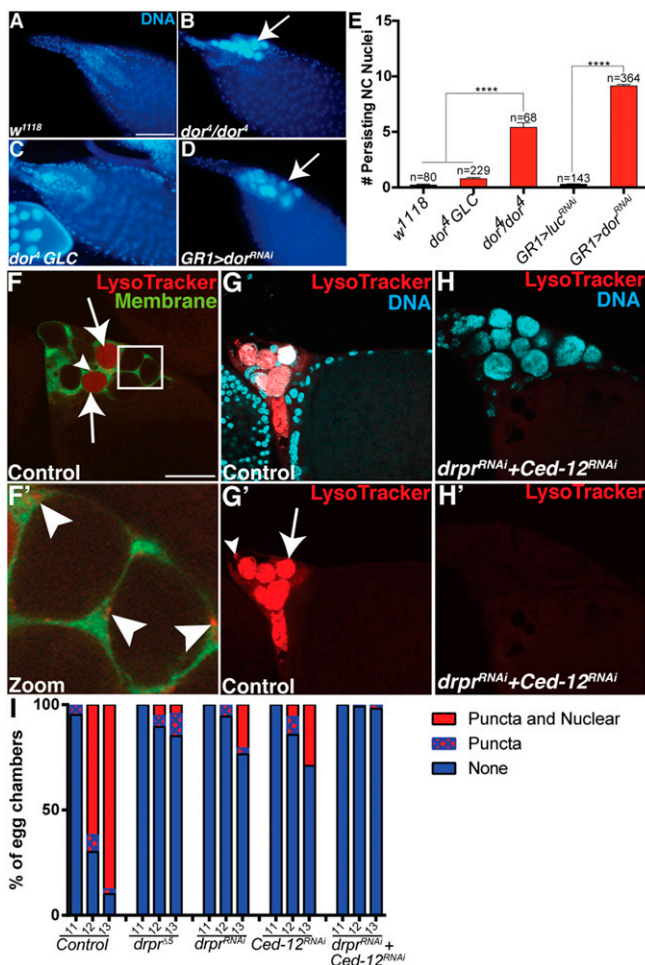


Fig. 3. The FC lysosomes play a role in NC removal. (A–D) *dor* is required in the FCs for NC removal. Stage 14 egg chambers were stained with DAPI (blue). (A) Control (*w¹¹¹⁸*) stage 14 egg chamber does not have persisting NC nuclei. (B) *dor* hypomorph (*dor^Δ/dor^Δ*) stage 14 egg chamber has persisting NC nuclei (arrow). (C) *dor^Δ* GLC stage 14 egg chamber is normal. (D) Knockdown of *dor* specifically in the FCs (*GR1-GAL4/UAS-dor^{RNAi}*) causes persisting nuclei (arrow). (Scale bar, 20 μ m.) (E) Quantification of persisting nuclei. *dor^{RNAi}* quantification includes two distinct RNAi lines. Data presented are mean \pm SEM. *****P* < 0.0001. (F and F') LysoTracker staining (red) on egg chambers that express membrane-GFP (green) specifically in stretch FCs (*PG150-GAL4/+; UAS-mCD8-GFP/+*). (Scale bar, 50 μ m.) (F) Two NC nuclei are acidified in a stage 12 egg chamber (arrows), and LysoTracker puncta are present within the stretch FC membranes (arrowhead). (F') Zoom of image pictured in F shows LysoTracker puncta in the stretch FC membranes. (G–H') Stage 13 egg chambers stained with DAPI (cyan) and LysoTracker (red). (G and G') Control (*UAS-GAL4/Sco; MKRS/TM6B*) stage 13 egg chamber has acidified NC nuclei (arrow) and LysoTracker puncta (arrowhead). (H and H') *draper Ced-12* double knockdown (*UAS-GAL4/UAS-draper^{RNAi}; GR1-GAL4 G89/UAS-Ced-12^{RNAi}*) stage 13 egg chamber does not contain acidified NC nuclei or LysoTracker puncta. (I) The percentage of late stage egg chambers (stages 11–13) with punctate or nuclear LysoTracker staining was quantified. Control is mixed siblings lacking either the GAL4 driver or the RNAi construct. Other genotypes are *draper^{Δ5}*, *draper^{RNAi}* (*UAS-GAL4/UAS-draper^{RNAi}; GR1-GAL4 G89/MKRS*), *Ced-12^{RNAi}* (*UAS-GAL4/Sco; GR1-GAL4 UAS-Ced-12^{RNAi}/GR1-GAL4 G89 or TM6B*), and *draper Ced-12* double knockdown (*UAS-GAL4/UAS-draper^{RNAi}; GR1-GAL4 UAS-Ced-12^{RNAi}/GR1-GAL4 G89 or TM6B*).

NC nuclear permeability, we used the *BB127 lacZ* enhancer trap that specifically labels NC nuclei (and centripetal FCs). Consistent with previous findings (54, 57, 58), we observed that control NC nuclei became permeable between stages 10B and 11, with β -Gal transitioning from a nuclear (Fig. 4 F and F') to cyto-

plasmic localization (Fig. 4 G and G'). However, *Ced-12^{RNAi}* and *draper^{RNAi} Ced-12^{RNAi}* double knockdowns displayed some stage 11 egg chambers with intact NC nuclei, suggesting that nuclear envelope permeability was delayed (Fig. 4 H–J). Eventually, all NC nuclei became permeable, indicating that that the genes were incompletely knocked down or that another pathway can promote NC nuclear permeabilization. These data suggest that the engulfment machinery in the FCs is important for the permeabilization of the NC nuclear envelope, an initial event during PCD. We also found that the NC nuclear lamina remained largely intact, surrounding the persisting nuclei in *draper* mutants (Fig. 4 K–N).

The Stretch FCs Are Required for NC Death and Dumping. To directly address whether the stretch FCs were required for the developmental PCD of the NCs, we eliminated the stretch FCs via the expression of RNAi against the caspase inhibitor *Diap1* (*PG150 > Diap1^{RNAi}*). The presence of pyknotic FC nuclei, cleaved caspase Dcp-1 staining, and TUNEL confirmed that the stretch FCs were indeed dying (Fig. 5 A–B' and Fig. S4 A–B'). The genetic ablation of the stretch FCs resulted in non-autonomous effects on the NCs: They failed to dump their cytoplasm into the oocyte or undergo developmental PCD (Fig. 5 C–D'). This disruption to NC dumping and PCD was nearly complete, with 98% of egg chambers exhibiting a failure in NC dumping and an average of 13 persisting nuclei per egg chamber (Fig. 5 K and L). Interestingly, we observed a correlation between increasing the time of *Diap1^{RNAi}* expression and the severity of egg chamber defects. Egg chambers expressing *Diap1^{RNAi}* for 4–10 h had increasing numbers of persisting NC nuclei, however egg chambers expressing *Diap1^{RNAi}* for 12–14 h had a mild dumplless phenotype that shifted to strong dumplless at 18 h (Fig. S4C). To further investigate which NC death events required stretch FCs, we stained *PG150 > Diap1^{RNAi}* egg chambers with LysoTracker and TUNEL. We observed that acidification (Fig. 5 E–F' and M) and DNA fragmentation (Fig. 5 G, H, and N) of the NCs were strongly inhibited. A dramatic event that occurs in wild-type egg chambers just before dumping is the formation of actin bundles in the cytoplasm (57) (Fig. 5I). Phalloidin staining revealed that these actin bundle networks failed to form when *Diap1^{RNAi}* was expressed in stretch FCs (Fig. 5J). These data demonstrate that the FCs nonautonomously control multiple events associated with the dumping and death of the NCs.

The JNK Signaling Pathway Is Required Specifically in the FCs for NC Removal. Previous work has demonstrated a role for the JNK pathway in the engulfment of apoptotic cells. For example, JNK is required in the FCs during engulfment of NCs in response to starvation during midoogenesis (27), in imaginal disc cells undergoing cell competition (59), and in glia during engulfment of degenerating axons (60). The JNK signaling pathway is activated in the anterior FCs during late oogenesis (61–63), but whether it plays a role in developmental PCD of the NCs was unknown. Therefore, we investigated whether the JNK pathway was an important component of signaling in the stretch FCs for developmental PCD of the NCs. Using the JNK reporter *puclacZ*, we confirmed that the JNK pathway was activated in the stretch FCs during late oogenesis (Fig. 6 A–D'). Up-regulation of the JNK pathway first became apparent in the FCs in stage 11 (Fig. 6 A and A' and Fig. S5A) and was most intense in stages 12 and 13 (Fig. 6 B–C'), with FCs showing *puclacZ* directly surrounding individual NC nuclei (Fig. 6 C and C'). NC death is not synchronous, and it may be that several stretch FCs work together to eliminate NCs one at a time. The activation of the JNK pathway paralleled the timing of the enrichment of Draper on the FC membranes (Fig. 1 F–J'), suggesting that JNK may also play an important role in NC removal.

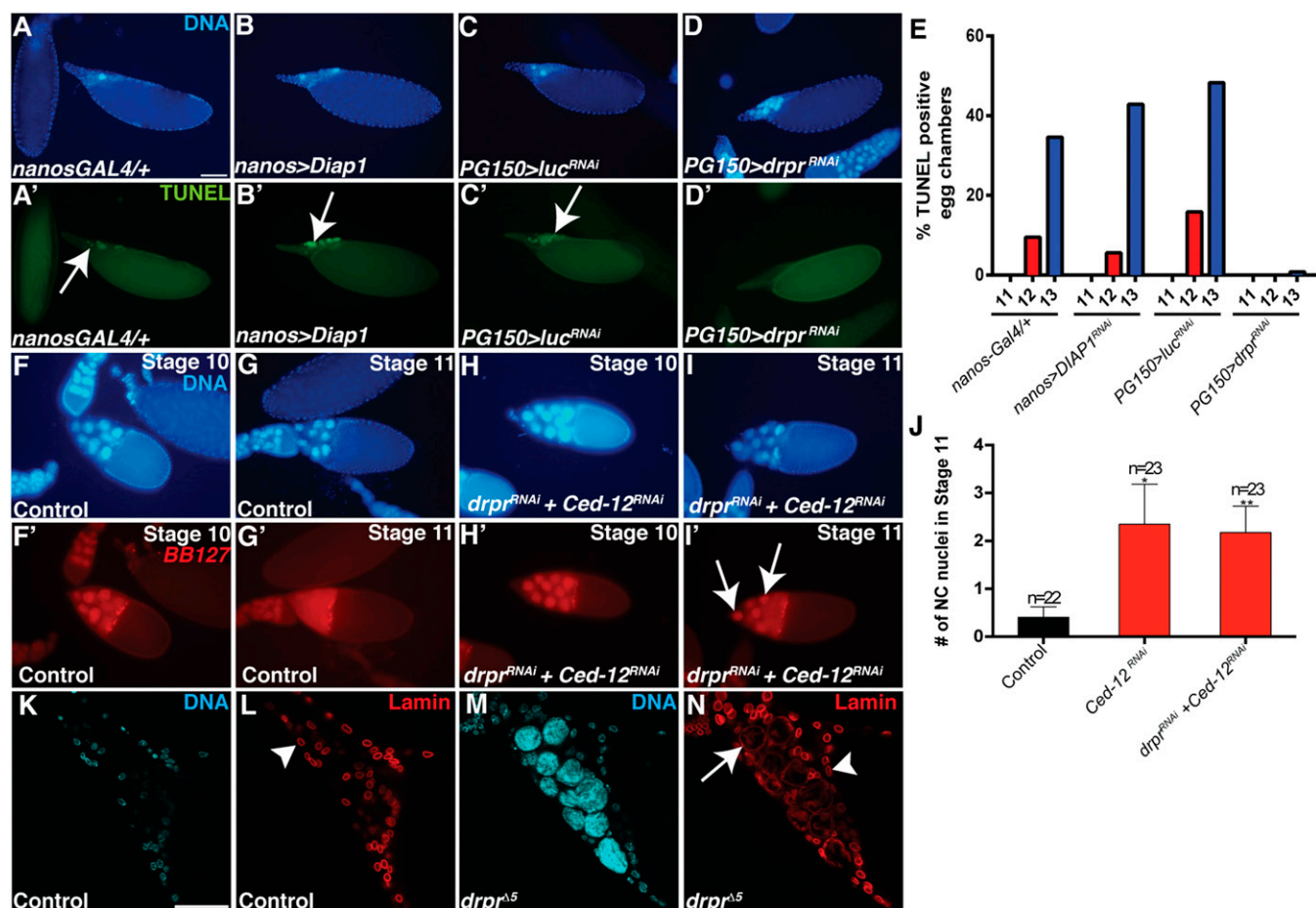


Fig. 4. The FCs nonautonomously contribute to NC death events. (A–D') Stage 13 egg chambers stained with DAPI (blue) and TUNEL (green) as a marker for DNA fragmentation. (Scale bar, 50 μ m.) (A and A') Control (*nanos-GAL4/+*) egg chamber contains several TUNEL-positive NC nuclei (arrow), although only one nucleus is visible by DAPI staining. (B and B') Overexpression of *Diap1* (*nanos-GAL4/UAS-Diap1*) shows several TUNEL-positive nuclei (arrow). (C and C') Control (*PG150-GAL4/+; UAS-luciferase^{RNAi}/+*) egg chamber has many TUNEL-positive NC nuclei (arrow). (D and D') The knockdown of *draper* specifically in the stretch FCs (*PG150-GAL4/+; UAS-draper^{RNAi}/+*) shows no TUNEL staining. (E) The percentage of egg chambers containing TUNEL-positive nuclei in the late stages of oogenesis (stages 11–13) was quantified for each genotype. (F–I') Egg chambers expressing the *BB127 lacZ* enhancer trap are stained with DAPI (blue) and α - β -Gal antibody (red) to detect nuclear leakage. *BB127* labels both NCs (large nuclei) and centripetal FCs (small nuclei). (F–G') Control egg chambers (mixed siblings, *BB127/+; Sco* or *UAS-draper^{RNAi}/CyO* or *UAS-GAL4; +/MKRS*) have NC nuclear β -Gal in stage 10B (F and F') and cytoplasmic β -gal in stage 11 (G and G'). (H–I') *draper Ced-12* double knockdowns (*BB127/+; UAS-draper^{RNAi}/CyO* or *UAS-GAL4; +/IGR1-GAL4 UAS-Ced-12^{RNAi}*) have nuclear β -Gal in stage 10B (H and H'). In this stage 11 egg chamber, β -Gal staining is still largely nuclear (arrows, I and I'). (J) The average number of intact NC nuclei in stage 11 egg chambers was quantified. Data presented are mean \pm SEM. * $P < 0.05$, ** $P \leq 0.01$. (K–N) Stage 14 egg chambers stained with DAPI (cyan) and Lamin Dm α ADL67.10 antibody (red). (K and L) Control egg chambers are devoid of NC nuclei, and Lamin staining appears at the periphery of remaining FC nuclei (L, arrowhead). (M and N) *draper Δ* mutant egg chambers have Lamin associated with persisting NC nuclei (N, arrow) in addition to labeling FC nuclei (N, arrowhead).

To test whether the JNK pathway was required in the FCs for elimination of the NCs, we inactivated several members of the JNK pathway. Expression of RNAi against *kayak* or *jra*, which encode components of the dimeric transcription factor AP-1, caused a strong persisting nuclei phenotype in stage 14 egg chambers (Fig. 6 E–G and I and Fig. S5B). Consistent with our findings, Dequier et al. previously reported that *kayak* hypomorphs contain persisting NC nuclei (63). Additionally, knockdown of the AP-1 scaffold *Cka* or overexpression of a dominant-negative form of *Drosophila JNK* (*basket*, *bsk*) led to persisting NC nuclei (Fig. 6 H and I). Together, these data suggest that the JNK signaling pathway is required in the FCs for NC removal.

Ced-12 Acts Upstream of JNK to Promote Draper Enrichment. During the engulfment of dying NCs by the FCs in midoogenesis in response to starvation, JNK was found to act downstream of Draper in a feed-forward loop to maintain Draper enrichment on the FC membranes (27). Similarly, during phagocytosis of

axonal debris by glia, JNK was required to increase Draper in glia to levels sufficient for clearance (60). Thus, we investigated whether the enrichment of Draper on FC membranes in late oogenesis required JNK activity. We found that *kayak* was not required for the initial localization of Draper on FC membranes in stage 11 (Fig. 7 A–B'), but it was necessary for enrichment of Draper in stages 12 and 13 (Fig. 7 C–D'). Thus, JNK signaling may act to maintain Draper on the FC membranes. These data are similar to previous findings showing that JNK is required for sustaining Draper levels in engulfing cells (27, 60). The inverse experiment examining JNK activity in *draper* mutants revealed that *draper* was not required for activation of JNK (Fig. S6 A–D'), unlike engulfment in midoogenesis (27). Interestingly, we found that *Ced-12* was required for the enrichment of Draper on FC membranes throughout late oogenesis (Fig. 7 E–H'). FC membranes in *Ced-12* knockdowns surrounded NCs normally (Fig. S1L); thus, defects in Draper enrichment were not due to morphological abnormalities in FCs.

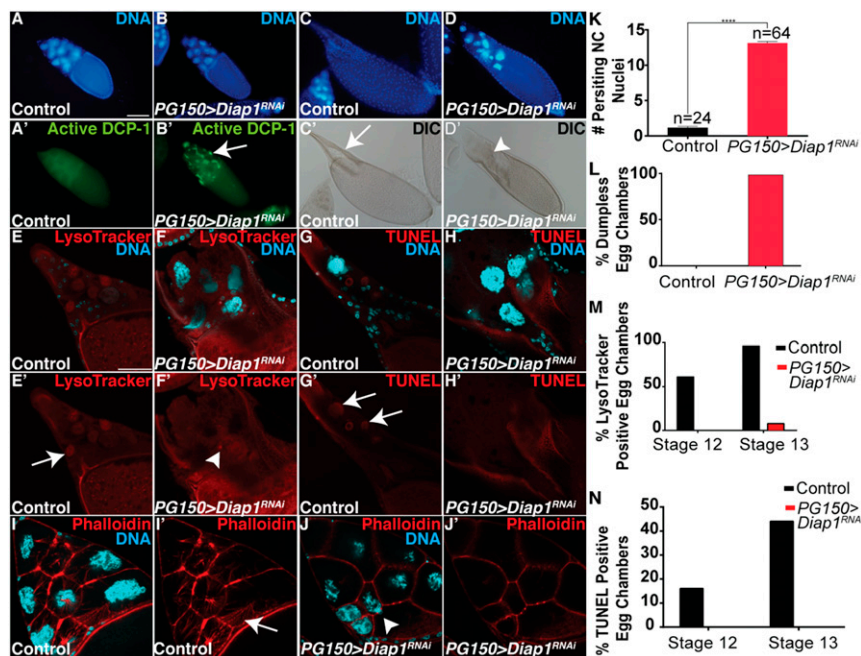


Fig. 5. The stretch FCs are required for NC dumping and developmental PCD. (A–B') Stage 10 egg chambers stained with DAPI (blue) and α -cleaved Dcp-1 (green). (A and A') Control (*FM7/+; tub-GAL80^{TS/+}; UAS-Diap1^{RNAi/+}*) has no detectable Dcp-1. (Scale bar, 50 μ m.) (B and B') Egg chamber expressing *Diap1^{RNAi}* in the stretch FCs (*PG150-GAL4/+; GAL80^{TS/+}; UAS-Diap1^{RNAi/+}*) exhibits α -cleaved Dcp-1 in stretch FCs (arrow). (C–D') Stage 14 egg chambers stained with DAPI (blue). (C and C') Control egg chamber has DAs and no persisting nuclei or NC cytoplasm (arrow). (D and D') Stage 14 *PG150 > Diap1^{RNAi}* egg chamber failed to undergo NC dumping (arrowhead). (E–F) Stage 13 egg chambers stained with DAPI (cyan) and LysoTracker (red). (E and E') Control egg chamber with nuclear LysoTracker staining (arrow). (Scale bar, 50 μ m.) (F and F') *PG150 > Diap1^{RNAi}* egg chamber with LysoTracker staining is absent in NC nuclei, although it is present in some FCs (arrowhead). (G–H') Stage 13 egg chambers stained with DAPI (cyan) and TUNEL (red). (G and G') Control egg chamber with TUNEL-positive NC nuclei (arrow). (H and H') *PG150 > Diap1^{RNAi}* egg chamber with no TUNEL-positive NC nuclei. (I–J') Stage 11 egg chambers stained with DAPI (cyan) and phalloidin (red). (I and I') Phalloidin labels cytoplasmic actin network in control egg chamber (arrow). (J and J') *PG150 > Diap1^{RNAi}* egg chamber lacks cytoplasmic actin network, and an NC nucleus is stuck in a ring canal (arrowhead). (K) Quantification of persisting NC nuclei. Data presented are mean \pm SEM. **** $P \leq 0.0001$. (L) Quantification of cytoplasmic dumping. (M) Quantification of LysoTracker staining. (N) Quantification of TUNEL.

Our data indicate that unlike midoogenesis (27), JNK is activated by a pathway distinct from Draper during late oogenesis. Indeed, we found that *Ced-12* is required to activate JNK: Egg chambers with *Ced-12* knocked down in the FCs showed reduced expression of *puc-lacZ* (Fig. 7 I–K). Taken together, we propose that *Ced-12* acts upstream of JNK, which leads to increased Draper in FCs to promote the removal of NCs.

Discussion

The nonautonomous control of PCD has wide-ranging implications. Before this study, the role of phagocytic machinery in promoting cell death has been documented in systems where phagocytosis is artificially activated (15, 27, 64) or acts in cooperation with apoptosis (17, 18, 65). In this work, we have demonstrated that a naturally occurring example of nonapoptotic PCD fails to occur properly when the phagocytic machinery is disrupted in surrounding cells.

In the adult female fly, hundreds of NCs die every day via developmental PCD, but previous work ruled out major roles for autonomous apoptosis and autophagic cell death mechanisms during late oogenesis (7, 9, 10, 55). We and others have previously shown that FCs can be genetically induced to perform phagocytosis during midoogenesis (27, 64). Therefore, we investigated whether the phagocytic machinery of the FCs might promote PCD and removal of NCs that occurs naturally during late oogenesis. Indeed, we found that there is a failure in NC removal when phagocytosis is disrupted in the somatic FCs that directly surround the NCs. We showed that the phagocytic genes *draper* and *Ced-12* act in at least two pathways to complete the process of NC removal in late oogenesis (Fig. S7), and we identified several other

genes that are important in the FCs for NC removal. We demonstrated that the events associated with the death of the NCs, including permeabilization of the nuclear envelope, acidification, lamin degradation, and DNA fragmentation, are impaired when phagocytosis genes are inhibited in the FCs. Furthermore, genetic ablation of the stretch FCs caused a near complete failure in NC death, emphasizing their central role in NC death. This work suggests that the FCs nonautonomously promote the death and removal of the NCs, likely via phagocytosis.

Although disruption of the phagocytic machinery largely prevented removal of the NCs, analysis of cellular events was necessary to determine whether the FCs affected the death of NCs. One indication that death of the NCs is nonautonomously controlled is that acidification of the NC nuclei is completely disrupted when *draper* and *Ced-12* were knocked down in the FCs. These data need to be interpreted carefully, as acidification is indicative not necessarily of cell death but perhaps degradation of a cell corpse. The presence of lysosomes within the stretch FCs and the block to acidification of NCs when phagocytosis is impaired in the FCs raises many questions about the role of the lysosomes in NC death. Lysosomes may be exocytosed from the FCs, releasing their contents to promote NC death. For example, T cells have been shown to exocytose lysosomes to destroy pathogens (66, 67). Alternatively, lysosomes could be delivered to the plasma membrane to allow for rapid membrane growth as the FCs stretch around the NCs (68). An open question is how FCs are capable of destroying the much larger NCs; perhaps stretch FCs fuse together to surround individual NCs. Our findings indicate that several stretch FCs surround the NCs and may cooperate to remove individual NCs in a stepwise manner. Previously, we found

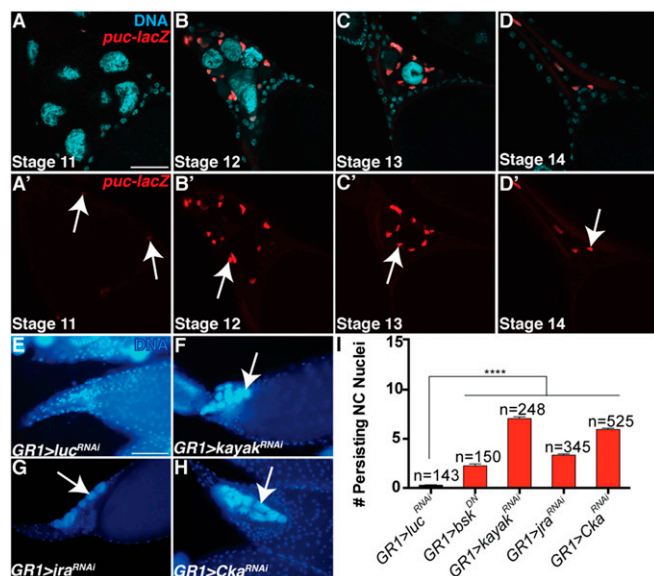


Fig. 6. JNK signaling is activated and required in the FCs during developmental PCD of the NCs. (A–D') *puc-lacZ/TM3* was used as a reporter for JNK activity (arrows). Stage 11–14 egg chambers are stained with α - β -Gal (red) and DAPI (cyan). *puc-lacZ* is initially detected in FC nuclei during stage 11 (A and A') and becomes most highly expressed in stages 12 and 13 (B and B' and C and C'). *puc-lacZ* is also apparent in some FCs along the DA in stage 14 (D and D'). (Scale bar, 50 μ m.) These data are quantified in Fig. S5A. (E–H) Persisting nuclei (arrows) are observed in stage 14 egg chambers of JNK pathway knockdowns stained with DAPI (blue). (E) *GR1-GAL4/UAS-luciferase^{RNAi}* control does not have persisting NC nuclei. (F–H) Persisting NC nuclei are found in (F) *GR1-GAL4/UAS-kayak^{RNAi}*, (G) *GR1-GAL4/UAS-jra^{RNAi}*, and (H) *GR1-GAL4/UAS-Cka^{RNAi}*. (Scale bar, 20 μ m.) (I) Quantification of persisting nuclei in JNK pathway knockdowns, including *bsk^{DN}*, *kayak^{RNAi}*, *jra^{RNAi}*, and *Cka^{RNAi}* (data from two distinct *Cka* RNAi lines were combined), compared with the control *GR1-GAL4/UAS-luciferase^{RNAi}*. Data presented are mean \pm SEM. **** $P \leq 0.0001$.

a requirement for *spinster* in the germ line during dumping and NC death, indicating that lysosomes may have both autonomous and nonautonomous roles (46). Further investigation is needed to clarify the role(s) of the lysosomes during NC death.

Further support for our conclusions that death of the NCs is nonautonomously controlled is that the NCs fail to become TUNEL-positive when the phagocytic machinery is disrupted in the FCs. In general, the initial steps of DNA fragmentation during PCD are thought to occur cell-autonomously; however, previous studies have indicated that DNA fragmentation can be carried out by engulfing cells. In mammals, apoptotic thymocytes from mice expressing caspase-resistant ICAD (inhibitor of CAD) were resistant to DNA fragmentation but became TUNEL-positive once engulfed by macrophages (69). One explanation is that lysosomal enzymes in engulfing cells may contribute to DNA fragmentation. Interestingly, it was demonstrated in *C. elegans* that *ced-1* engulfment mutants had a reduced number of corpses with TUNEL-positive nuclei (70). In the ovary, we found that DNA fragmentation was strongly disrupted in wild-type NCs when phagocytosis genes were inhibited in the FCs. The Nomenclature Committee on Cell Death has suggested that cells should only be considered dead when they “either exhibit irreversible plasma membrane permeabilization or have undergone complete fragmentation” (1). We found that many NC nuclei remained intact in engulfment mutants, retaining their nuclear lamina and failing to undergo DNA fragmentation, indicating that death was disrupted.

The first indication that the NCs are dying occurs between stages 10 and 11 when the NC nuclei become permeable (57). This

event is clearly separable from engulfment, as it occurs before the onset of dumping and envelopment by FCs. We found that permeability of the NC nuclear envelope was delayed when *draper* and *Ced-12* were knocked down specifically in the FCs. Ultimately, the NC nuclei did become permeable, suggesting that other pathways can also promote NC nuclear permeabilization. These other pathways are likely to initiate from the stretch FCs, as ablation of the stretch FCs blocked all NC death.

Ablation of the stretch FCs caused a failure in NC dumping, which occurs concurrently with NC death. Many genes have been

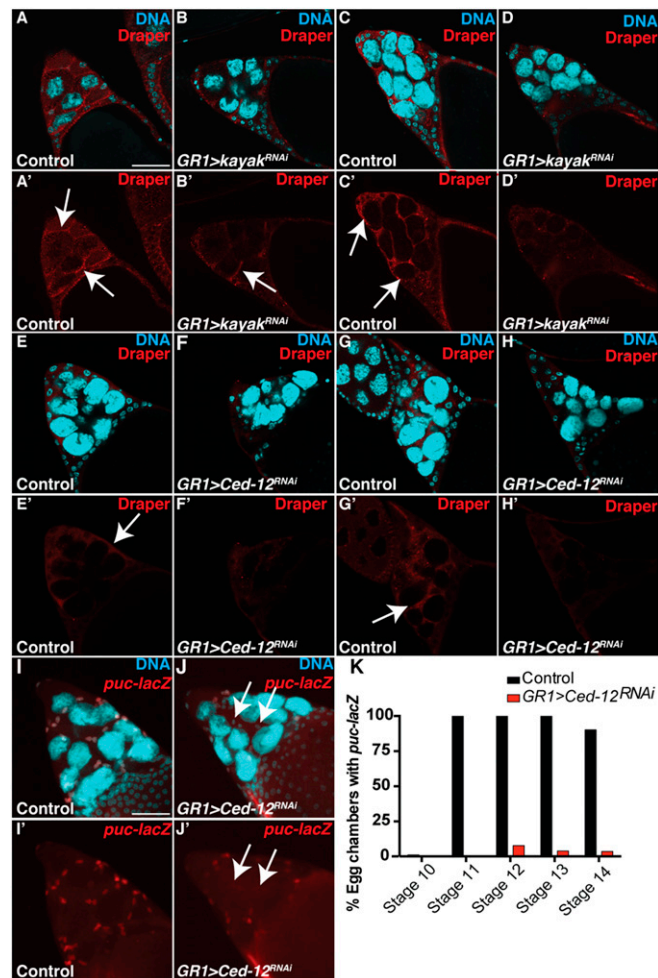


Fig. 7. *Ced-12* acts upstream of JNK in late oogenesis. (A–D') Control (*PG150-GAL4/+; UAS-mCD8-GFP/+*) and *kayak* knockdown (*GR1-GAL4/UAS-kayak^{RNAi}*) egg chambers were stained with α -Draper antibody (red) and DAPI (cyan). (Scale bar, 50 μ m.) (A–B') The initial expression of Draper during stage 11 is unaffected. (C–D') The enrichment of Draper on the FC membranes in *kayak* knockdown in stage 12 (D and D') is reduced compared with the control (C and C'). (E–H') Control (*GR1-GAL4/UAS-mCD8-GFP*) and *Ced-12* knockdown (*GR1-GAL4/UAS-Ced-12^{RNAi}*) egg chambers were stained with α -Draper antibody (red) and DAPI (cyan). (E–F') The initial enrichment of Draper on the FC membranes during stage 11 is not apparent in the *Ced-12* knockdown. (G–H') The enrichment of Draper on the FC membranes in the *Ced-12* knockdown in stage 12 (H and H') is absent compared with the control (G and G'). (I–J') Projection images (34 slices) of stage 11 egg chambers stained with DAPI (cyan) and α - β -Gal (red) as a readout of JNK activity. (I and I') Most stretch FCs in control *puc-lacZ/TM3* egg chambers express β -Gal. (J and J') *puc-lacZ/GR1-GAL4 UAS-Ced-12^{RNAi}* egg chamber FCs have reduced β -Gal expression (arrows). (K) Quantification of *puc-lacZ* in *Ced-12* knockdowns (*GR1-GAL4 Ced12^{RNAi}/puc-lacZ*) compared with the control (*puc-lacZ/TM3*). Egg chambers were considered to have activated *puc-lacZ* when >10 FCs were stained with α - β -gal.

identified as important for NC dumping, particularly genes affecting the ring canals and actin cytoskeleton (71). For example, *spaghetti squash* encodes the regulatory light chain of myosin II and must be phosphorylated to promote NC dumping. It has been proposed that the FCs release a signal that activates the kinase that phosphorylates regulatory light chain of myosin II (72). Although both NC death and dumping were disrupted when the FCs were ablated, it is unlikely that dumping is the signal for death because NC nuclear permeability, a cell death event, is apparent before the onset of dumping. Furthermore, NCs in other dumpless mutants (e.g., *chickadee*) have TUNEL-positive NC nuclei (55, 56). It remains to be determined how the stretch FCs promote the precise developmental timing of NC death. Moreover, how the FCs become activated to promote dumping and remove NCs is not clear; an upstream signal could originate from the oocyte, in the NCs, or from a source extrinsic to the ovary.

Although our experiments aimed at disrupting the phagocytic machinery in the FCs caused a severe block to NC death and removal, it did not cause a total block. When *draper* and *Ced-12* were knocked down in the FCs, there was an average of ~11 persisting NC nuclei, meaning that on average four NCs per egg chamber were successfully eliminated. Knockdown of integrins specifically in the stretch FCs also led to persisting nuclei, raising the possibility that integrin signaling and/or other pathways act in parallel to *draper* and *Ced-12*. Alternatively, some cells that fail to be removed by the normal phagocytic mechanism could die by another pathway such as necrosis. Although previous work has ruled out some autonomous mechanisms including apoptosis and autophagic cell death as major contributors to NC death, mutants do show weak phenotypes, suggesting that these processes play a minor role. Furthermore, there are likely unknown cell-autonomous effectors of developmental NC death.

Previously we determined that Draper acted upstream of JNK in FCs for the phagocytosis of NCs that die in midoogenesis in response to starvation (27). Our findings here differ in several ways. First, we have found that phagocytosis genes are required for NC death in addition to clearance in late oogenesis, whereas the role is limited to clearance in midoogenesis. Second, we have found that Draper does not regulate JNK activity in late oogenesis, in contrast to midoogenesis. Moreover, we have found that *Ced-12* acts upstream of JNK, leading to increased Draper protein, in late oogenesis (Fig. S7). However, our double mutant analysis (Fig. 2) indicates that *Ced-12* also has a role in NC removal that is independent of Draper. These findings suggest that the roles of JNK, *Ced-12*, and Draper may differ depending on whether the cell is promoting phagoptosis (cell death) or phagocytosis (clearance).

Germ-line cysts are found in the ovary and testis of many organisms. Interestingly, non-cell-autonomous control over cell death of the germ line has been suggested to occur in several species. For example, the somatic sheath cells of the *C. elegans* gonad promote apoptotic death of the germ cells in conjunction with autonomous cell death mechanisms (65). In the developing mammalian ovary, germ-cell cysts break apart and oocytes become surrounded by somatic cells. At the same time, ~2/3 of the oocytes undergo cell death. Deletion of *Notch2* in the somatic cells causes reduced oocyte death and defects in the breakdown of germ-cell cysts, resulting in reduced fertility (73). Similarly, we found that failure of the NCs to appropriately undergo PCD reduced egg laying of *Drosophila* females. The exact mechanism

of how a failure in developmental PCD of the NCs leads to reduced egg laying is unknown. It is possible that accumulation of NC nuclei blocks the entrance to the oviducts, or there could be feedback signaling to the stem cells that affects the rate of egg production. Therefore, non-cell-autonomous PCD in the germ line may be evolutionarily conserved and play a critical role in the reproductive success of organisms. Overall, we demonstrated that the phagocytosis machinery in FCs promotes the death and removal of NCs during the late stages of *Drosophila* oogenesis. To our knowledge, this is the first example of developmental PCD that is both nonapoptotic and non-cell-autonomously controlled.

Materials and Methods

Fly Strains and Manipulations. Unless otherwise indicated, flies were obtained from the Bloomington Stock Center and raised at 25 °C on standard cornmeal/molasses food. RNAi and dominant-negative lines are listed in Table S1. *draper^{Δ5}*, a null allele (22), was provided by Estee Kurant, Technion Israel Institute of Technology, Haifa, Israel. All recombinants were confirmed by PCR. *PWIZ-draper^{RNAi} #7b* (23) was provided by Mary Logan and Marc Freeman, University of Massachusetts, Worcester, MA. *Ced-12^{KO}*, a null allele (74), was provided by Erika Geisbrecht, Kansas State University, Manhattan, KS. *dor^Δ* is a partial loss-of-function allele (47). *GRI-GAL4* (75) was used to drive expression specifically in all FCs after stage 3 (27) and was provided by Trudi Schüpbach, Princeton University, Princeton. *PG150-GAL4* (76) was used to drive expression specifically in the stretch FCs and was provided by Ellen LeMosy, Augusta University, Augusta, GA (77). Typically, *GRI-GAL4* was used for experiments so that RNAi was expressed in FCs for a longer time during egg chamber development. *PG150-GAL4* was used when we wished to limit expression to stretch FCs. GLCs were generated using the *ovo^D* method as described (78). *nanos-GAL4* was used to drive the overexpression of *UASp-Diap1* specifically in the germ line (7). The reporter for JNK activity was *puc-lacZ^{A251.1F3} ry/TM3* (79). To obtain well-developed ovaries, females <20 d old (unless otherwise noted) were conditioned on fresh yeast paste for at least 48 h, changing yeast paste every 24 h. See *SI Materials and Methods* for more details.

Staining Methods. See *SI Materials and Methods* for more details. Standard antibody staining techniques were used as described in ref. 53. LysoTracker Red DND-99 (1:50, Invitrogen) was used to detect acidified compartments (49). We used the DeadEnd Fluorometric TUNEL system (Promega) (53). Samples were mounted in VectaShield with DAPI (Vector Labs) and imaged on an Olympus FV10i confocal microscope or Olympus BX60 upright fluorescence microscope. Images were processed and compiled in Image J, Adobe Photoshop, and Adobe Illustrator.

Quantifications and Statistics. We used GraphPad Prism to graph and analyze all of our data. The unpaired *t* test was used for all statistical analyses. Persisting NC nuclei (PN) in stage 14 egg chambers were quantified in “bins” of 0 PN, 1–3 PN, 4–6 PN, 7–9 PN, 10–12 PN, or 13–15 PN and presented as a percentage of total stage 14 egg chambers. To calculate the average number of PN, the median number in each bin (e.g., two in the 1–3 bin) was used. With the exception of fecundity analyses, “n” always refers to the number of egg chambers quantified. To quantify NC nuclear permeability, the number of NC nuclei that were still intact in stage 11 were counted.

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